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03/30/94

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application	: Daniel CAPUT et al.
Serial N°	: 07/920,519
Filed	: July 28, 1992
For	: URATE OXIDASE ACTIVITY PROTEIN, RECOMBINANT GENE CODING THEREFOR, EXPRESSION VECTOR, MICROORGANISM AND TRANSFORMED CELLS
Examiner	: David SCHMICKEL
Group Art Unit	: 1814

DECLARATION UNDER 37 C.F.R § 1.132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir :

The undersigned, Gérard LOISON, a French citizen, declares as follows :

1. I obtained a PhD in microbiology at the University Pierre et Marie Curie (Paris, France) in 1979 and an Official Doctor's Degree at the University Louis Pasteur (Strasbourg, France) in 1982 (see appendix A). I have been employed by SANOFI, now named ELF SANOFI, since January 1, 1988 as Head of the Laboratory "Expression of Microorganisms" from January 1988 to January 1989 and as Head of the Microbiology Department

since then. I am co-author or co-inventor of the articles and patent applications listed in appendix A.

2. I am a co-inventor of the above-captioned patent application.

3. I have reviewed Reddy et al., Proc. Natl. Acad. Sci. USA 85 : 9081-9085 (1988), a reference cited in the rejection of the claims of the captioned application. The following statements are formed upon my knowledge of this document and my personal knowledge of the field.

4. Reddy et al. relates to the isolation and sequence determination of a cDNA clone for rat peroxisomal urate oxidase. More precisely, a cDNA clone for urate oxidase containing an insert of 1.3 kb is isolated from a λ gt 11 cDNA library prepared from rat liver RNA. The isolation of the mRNAs is performed according to the method described by Chirgwin et al., Biochemistry 18 : 5294-5299 (1979), attached to this declaration as Appendix B. This method involves in particular dissociating animal cell tissues and suspending the dissociated tissues in guanidinium thiocyanate. Thus, in Reddy et al., rat liver cell tissues are dissociated and then suspended in guanidinium thiocyanate, which is sufficient to lyse these cell tissues, which are devoid of walls, and then to free the mRNAs.

5. Such a technique cannot however be applied for the isolation of mRNAs from <u>A.flavus</u> : the dissociation of animal cells, as described by Chirgwin et al., *supra*, does not work with fungal cells because contrary to animal cells, fungal cells do have walls. More particularly, <u>A.flavus</u> has a polysaccharide mycelian wall. The mere dissociation and suspension steps

recited above will therefore not allow the recovery of mycelium from culture of A.flavus. The isolation of mRNAs from A.flavus thus requires a different technique, which has been specifically designed for the present invention and which is described at page 12 of the specification : the mycelium must be frozen in liquid nitrogen, thawed and suspended in lysis buffer, ground with beads to break the mycelium and free the mycelian extract. Due to the great number of polysaccharide particles - corresponding in particular to wall residues - the mRNAs must be then selectively precipitated in the presence of lithium chloride.

6. As explained, the RNA isolation technique described by Chirgwin et al., and used by Reddy et al. to extract mRNAs coding for rat liver urate oxidase, does not make it possible to isolate mRNAs coding for A.flavus urate oxidase.

7. I, hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true ; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 ot Title 18 of the United States code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

GLoison

03/08/94 Gérard Loison.

Date Gérard LOISON

APPENDIX A

GERARD LOISON

EDUCATION

1979 : PhD degree at the University Pierre et Marie Curie (Paris, France).
Subject : Study of the regulation of yeast (*S. cerevisiae*) URA3 gene.

1982 : Official's Doctor degree at the University Louis Pasteur (Strasbourg, France).
Subject : Expression of *S. cerevisiae* URA1 gene in homospecific and heterospecific environment.

PUBLICATIONS AND PATENT APPLICATIONS

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Isolation of Biologically Active Ribonucleic Acid from Sources Enriched in Ribonuclease†

John M. Chirgwin,‡ Alan E. Przybyla,§ Raymond J. MacDonald,∥ and William J. Rutter*

ABSTRACT: Intact ribonucleic acid (RNA) has been prepared from tissues rich in ribonuclease such as the rat pancreas by efficient homogenization in a 4 M solution of the potent protein denaturant guanidinium thiocyanate plus 0.1 M 2-mercaptoethanol to break protein disulfide bonds. The RNA was isolated free of protein by ethanol precipitation or by sedimentation through cesium chloride. Rat pancreas RNA obtain by these means has been used as a source for the purificati of α-amylase messenger ribonucleic acid.

The preparation of undegraded ribonucleic acid from a number of cell types is hindered by the presence of active nucleases. An extreme example of this is the rat pancreas which contains over 200 μg of ribonuclease A per g of tissue wet weight (Beintema et al., 1973). Within the pancreatic exocrine cells, ribonuclease A as well as other digestive enzymes and zymogens appears to be synthesized on ribosomes bound to the cytoplasmic face of the endoplasmic reticulum, extruded directly into the cisternal side, and subsequently packaged in secretory granules. Thus, the functions of the cytosol are effectively sequestered from these strong hydrolytic activities. Disruption of the cells, however, inevitably results in rapid mixing of RNA and RNase.[1,2] One way to eliminate nucleolytic degradation of RNA is to denature all of the cellular proteins including RNase. This approach would be successful only if the rate of denaturation exceeds the rate of RNA hydrolysis by RNase. Deproteinization procedures using guanidine hydrochloride (Cox, 1968) or phenol even in the presence of RNase inhibitors such as heparin, iodoacetate, and detergent (Parish, 1972) are insufficiently effective to yield intact RNA from the pancreas.

We describe here a generally applicable method for quantitative isolation of intact RNA. The rate of denaturati is maximized by the combined use of a strong denatura guanidinium thiocyanate, in which both cation and anion potent chaotropic agents (Jencks, 1969), and a reductant break protein disulfide bonds which are essential for RN activity (Sela et al., 1956). This method has been employ in the isolation of intact biologically functional RNA from pancreas and the purification of mRNA for α-amylase, most abundant pancreas-specific protein (Sanders & Ru 1972).

Experimental Procedure

Chemicals and Solutions. All glassware was rendered clease free by overnight treatment at 180 °C. Whene possible [see Ehrenberg et al. (1974)], stock solutions w treated for 20 min with 0.2% diethyl pyrocarbonate and thoroughly boiled to remove traces of the reagent. Buff such as tris(hydroxymethyl)aminomethane, which contai primary amine that reacts with diethyl pyrocarbonate, avoided.

Guanidinium thiocyanate stock (4 M) was prepar mixing 50 g of Fluka purum grade guanidinium thiocy (Tridom, Inc., Hauppage, NY) with 0.5 g of sodium N royisarcosine (final concentration 0.5%), 2.5 mL of 1 M dium citrate, pH 7.0 (25 mM), 0.7 mL of 2-mercap (0.1 M), and 0.33 mL of Sigma 30% Antifoam A (0.

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[1] A brief note describing a version of this method has been pu (Ullrich et al., 1977).

[2] Abbreviations used: RNase, ribonuclease; mRNA, messeng nucleic acid; cDNA, complementary deoxyribonucleic acid.

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…d water was added with warming and stirring until …ume equaled 100 mL at room temperature. The solu… which contained some insoluble material, was filtered, …H was adjusted to 7 with a small amount of 1 N NaOH, …d it was stored tightly closed for up to 1 month at room …perature. All handling of this solution and the initial …mogenization were done in a fume hood, and all equipment which came into contact with 2-mercaptoethanol was subse…ently treated with dilute aqueous hypochlorite solution (chlorine laundry bleach).

Guanidine hydrochloride (Sigma practical grade) was made up to 7.5 M, filtered, neutralized to pH 7.0, buffered with 0.025 volume of 1 M sodium citrate, pH 7.0, made 5 mM in either dithiothreitol or dithioerythritol, and stored for up to 1 month at room temperature.

Standard Guanidinium Thiocyanate Extraction Procedure. Freshly removed pancreases were trimmed free of lymph nodes, ganglia, and fat, weighed (the pancreas from a 300-g female rat weighs ~1 g), and then individually dropped into 16 mL of guanidinium thiocyanate stock solution in a 55-mL Potter–Elvehjem homogenizer tube and immediately homogenized for 30–60 s at full speed with an 18-mm diameter Tissumizer homogenizer (Tekmar Industries, Cincinnati, OH). The homogenates of two pancreases were combined in a 50-mL tube and centrifuged for 10 min at 8000 rpm in a Sorvall HB4 swinging bucket rotor at 10 °C to sediment particulate material. The supernatants were decanted into a flask and mixed with 0.025 volume (relative to the original volume of homogenizing buffer) of 1 M acetic acid to lower the pH from 7 to 5 and 0.75 volume of absolute ethanol. The flask was capped, shaken thoroughly, and placed at −20 °C overnight to precipitate nucleic acid. The material was sedimented by centrifugation for 10 min at −10 °C and 6000 rpm in an HB4 rotor. The tubes were drained of supernatant and any material which did not form a firm pellet. The pellet was then resuspended by vigorous shaking in 0.5 volume (relative to the original volume of homogenization buffer) of buffered guanidine hydrochloride stock solution. If necessary, the samples were briefly warmed in a 68 °C water bath to ensure complete dispersion of the pellets. RNA was reprecipitated by adding (relative to the amount of guanidine hydrochloride) 0.025 volume of 1 M acetic acid and 0.5 volume of ethanol. The solution was kept for at least 3 h at −20 °C and centrifuged as before. A final reprecipitation from guanidine hydrochloride was performed in the same way, with a further halving of the total volume. This reprecipitated material was centrifuged for only 5 min at 6000 rpm. From this point onward all procedures were carried out under sterile conditions to prevent nuclease contamination.

The final pellets were dispersed in ethanol at room temperature, triturated if necessary to extract excess guanidine hydrochloride, and again centrifuged for 5 min at 6000 rpm. Ethanol was removed from the pellet by a stream of nitrogen, and the RNA was dissolved with vigorous shaking in 1.0 mL of sterile water per g of original tissue. This solution was centrifuged for 10 min at 13 000 rpm and 10 °C to sediment insoluble material. The supernatants containing the RNA were decanted and saved, while the insoluble material was …tracted twice with 0.5 mL of sterile water per g of original …ue wet weight, followed by centrifugation for 10 min at …000 rpm and 10 °C. The combined aqueous solution was …ed with 0.1 volume of 2 M potassium acetate, pH 5, and …umes of ethanol and left overnight at −20 °C.

…RNA was sedimented from the ethanol suspension by cen…ation for 20 min at 10 000 rpm and −10 °C in Corex tubes in an HB4 rotor. The pellets were thoroughly washed with 95% ethanol, dried with nitrogen, and dissolved in 1.0 mL of sterile water per g of starting tissue. Absorbance measurements were obtained by diluting the RNA solutions into 10 mM triethanolamine hydrochloride, pH 7.4. An $E_{1cm}^{1\%}$ of 200 at 260 nm was used to determine the concentration of RNA. The RNA samples were routinely stored as 70% ethanol suspension at pH 5 and −20 °C.

All of the extraction procedures were routinely carried out in polyethylene, polypropylene, or Corex centrifuge tubes. Exposure to guanidinium thiocyanate solutions resulted in a high rate of failure for polycarbonate tubes.

In view of the large amounts of ribonuclease in the guanidine homogenates and supernatants, it was imperative that no contamination of any glassware or chemicals by these solutions be permitted. Dilution of solutions of denatured RNase results in renaturation of the active enzyme (Sela et al., 1956).

A modification to the above procedure in which the RNA is separated from the guanidinium thiocyanate homogenate by ultracentrifugation through a dense cushion of cesium chloride (Glisin et al., 1974) was suggested by Dr. A. Ullrich. For this procedure, technical grade cesium chloride was made up to 5.7 M, buffered with 0.1 M sodium ethylenediaminetetraacetate, pH 7, or 25 mM sodium acetate or citrate, pH 5, sterilized with 0.2% diethyl pyrocarbonate, and passed through a 0.45-μm Millipore filter. Small amounts of tissue were homogenized in 4 M, filtered guanidinium thiocyanate with a small Tissumizer or Potter–Elvehjem and layered into ultracentrifuge tubes one-quarter filled with 5.7 M cesium chloride. In a typical experiment, a Beckman SW50.1 rotor was centrifuged for 12 h at 36 000 rpm and 20 °C. Dissolution of the RNA pellets in water was facilitated by brief heating in a 68 °C water bath or by first extracting excess cesium chloride with ethanol and drying with nitrogen. Since the supernatants in these experiments could contain large amounts of renaturable nuclease, great care was taken not to contaminate the pellets during their dissolution. This danger could be circumvented by suspending the pellets in a small volume of buffered guanidine hydrochloride stock solution and precipitating the RNA with ethanol as described above. Cesium chloride has been used for the preparation of samples of less than 100 μg of embryonic RNA (Harding et al., 1978) and for the isolation of rat parotid RNA free from polysaccharides (Swain and Rutter, unpublished experiments). When the maximum rotor speeds permissable for dense cesium chloride solutions are calculated, allowance must be made for the specific gravity of the guanidinium thiocyanate homogenates, which is between 1.1 and 1.2 g/mL.

Procedural Anecdotes and Variations. A large number of different experimental procedures were tested before reaching those described above. A summary of our experience is given here to facilitate adaptation of the procedure to other systems. First, the prevention of degradation by ribonuclease is dependent upon the efficiency of the initial seconds of the homogenization. For this reason, we have used the high-speed Tissumizer; the similar Polytron (Brinkmann Instruments, Westbury, NY) undoubtedly would be satisfactory. The use of a conventional blender or the homogenization of tissue which has been frozen and thawed or of tissue which has been pulverized in liquid nitrogen results in degradation of the RNA, as detected by the diminution in the 28S peak height and the concomitant appearance of lower molecular weight species on electrophoresis in denaturing gels. However, rat pancreases which have been lyophilized after pulverization in dry ice or liquid nitrogen can be satisfactorily extracted with the guani-

dinium thiocyanate procedure. For tissues lacking significant nucleolytic activity, conditions for homogenization are less stringent. Embryonic pancreases can be prepared with a small Potter–Elvehjem homogenizer, and for some cultured cells no homogenization is needed since the cells lyse upon addition of the guanidine solution (Harding et al., 1977; Strohman et al., 1977).

The reprecipitations of the RNA aid to eliminate already denatured ribonuclease from the nucleic acid pellets. Thus, these steps can be varied according to the specific circumstances. The pH and temperature of the initial homogenization, 7.0 and ~20 °C, are optimal, but some variations can be tolerated. The use of the detergent sodium *N*-lauroylsarcosine is not essential but gives a cleaner initial precipitate of RNA and may accelerate the initial dissolution of the tissue. The 2-mercaptoethanol is essential for tissue containing RNase, but increasing its concentration beyond 0.1 M final concentration has no effect. Dithiothreitol can be used with the guanidine hydrochloride stock as a disulfide bond reductant, but it undergoes a chemical reaction with the thiocyanate anion to produce hydrogen sulfide and a green color.

The use of pH 7 and room temperature to dissolve the RNA and of pH 5, −20 °C, and the addition of 0.5 volume of ethanol to precipitate it follow the recommendation of Cox (1968) for guanidine hydrochloride. It is essential to determine empirically the time necessary for complete precipitation of a given RNA sample at −20 °C [viz., Strohman et al. (1977)]. Cooling to this temperature can be accelerated by the use of a 3:1 crushed ice–rock salt bath. It is also advisable to maintain RNA concentrations above 25 µg/mL in guanidine solution. Tissue can be homogenized in as little as 4 volumes of 4 M guanidinium thiocyanate, but the resultant solution may be too viscous to permit easy sedimentation of the RNA. The initial precipitation described above uses 0.75 volume of ethanol relative to guanidinium thiocyanate stock; this precipitates some DNA (eliminated by the reprecipitations) as well as RNA (Cox, 1968) but is necessary to prevent guanidinium thiocyanate from crystallizing out of solution at −20 °C. It is convenient to decrease the volumes of the successive precipitations to concentrate the RNA. Inclusion of a final organic solvent extraction step, for example, with phenol or chloroform, or of a 3 M sodium acetate precipitation at pH (Kirby, 1968) is unnecessary.

Some tissues may contain non-RNA molecules which coprecipitate with RNA by the methods described, necessitating further purification. We have, however, not encountered such contaminants in the tissues listed in Figure 2 or in rat brain, spleen, or muscle. Under the described conditions of centrifugation, yeast ^{3}H-labeled tRNA (provided by Dr. L. De Gennaro) was not sedimented. Similarly, in the standard procedure tRNAs (and DNA) are not precipitated from guanidine hydrochloride plus 0.5 volume of ethanol, as noted by Cox (1968).

Since the early steps of the procedure are always carried out in the presence of denaturants, sterile procedures and glassware are unnecessary, but as soon as the RNA is no longer in the presence of guanidine, stringent precautions against adventitious nucleases must be taken.

Preparation of Polyadenylated RNA. Polyadenylated species were separated from rRNA by two cycles of binding to oligo(dT)–cellulose (Type T-2, Collaborative Research, Waltham, MA). The procedure of Aviv & Leder (1972) was modified by the use of 0.5 M lithium chloride, 0.2% dodecyl sulfate, and 10 mM triethanolamine hydrochloride, pH 7.4, as the binding buffer. For minimization of nonspecific ribosomal contamination of the polyadenylated RNA, the sam[ples] were heated for 2 min at 68 °C at a concentration of no [more] than 2.5 mg/mL in sterile water and then rapidly quen[ched] on ice immediately prior to the other additions listed a[bove] and application to the column. Bound polyadenylated R[NA] was eluted with 10 mM triethanolamine hydrochloride, [pH] 7.4, without an intermediate 0.1 M salt wash.

Resolution Analysis of Isolated RNA Species. The mR[NA] preparations were subjected to gel electrophoresis in 3% a[ga]rose, 6 M urea, and 25 mM sodium citrate, pH 3.5, accordi[ng] to a modification of the procedure of Woo et al. (197[5]). Agarose was dissolved in buffered 6 M urea plus 0.02% An[]tifoam A by holding in a boiling water bath until uniformly in solution and free of bubbles. The solution was poured at 30 °C and allowed to gel overnight at 5 °C. Cylindrical gels were removed partially from their tubes, cut into 98-mm lengths, returned to the tubes, and held in place with gauze. After application of a 20-µg sample to a 5-mm diameter gel in buffered urea plus 10% sucrose, electrophoresis was conducted for 4 h at 100 V and room temperature with rapid recirculation of reservoir buffer (25 mM citrate, pH 3.5). The gels were washed for at least 1 h in sterile 25% glycerol and scanned at 260 nm.

For analysis of electrophoretically resolved mRNAs, agarose gels were cut with a razor blade immediately after being scanned and the slices were extracted 3 times each with [a] volume of oligo(dT)–cellulose binding buffer equal to the volume of the gel slices at room temperature for 24 h. RNA was recovered from the combined extracts by ethanol precip[]itation. The samples were dissolved in water, centrifuged t[o] remove particles of agarose, and analyzed by translation either directly or after rebinding to and elution from oligo(dT)–cel[]lulose.

The RNA preparations were tested for biological activi[ty] by translations in a cell-free system as described by MacDo[n]ald et al. (1977) after nuclease pretreatment according t[o] Pelham & Jackson (1976). In addition, cDNA was prepare[d] from these samples and hybridization analyses were performe[d] as described by Harding et al. (1977).

Results

Our interest in specific pancreatic genes and their expressio[n] has led to rather extensive investigations of methods for pre[p]aration of biologically functional RNA from tissues rich i[n] RNase. A number of methods were tested: some were usefu[l] but none were found to be completely satisfactory.

An extensive analysis of the hybridization characteristic[s] of rat pancreas RNA and cDNA made from it with revers[e] transcriptase has been published by Harding et al. (1977). These experiments were conducted with RNA prepared by [a] precursor to the present procedure, one in which the tissue[s] were homogenized in 7.5 M guanidine hydrochloride plus 1[%] diethyl pyrocarbonate (Zsindely et al., 1970). This procedu[re] gave undegraded RNA which, however, appeared to be par[]tially modified by the diethyl pyrocarbonate (Ehrenberg et al., 1974). When rat pancreas RNA prepared with guanidi[ne] hydrochloride plus diethyl pyrocarbonate was translated i[n] vitro, there was a marked lower efficiency of synthesis of high[er] molecular weight polypeptides, especially amylase. This effe[ct] was much more pronounced in the reticulocyte lysate than i[n] the wheat germ cell-free system of Roberts & Paterson (1973). Attempts to circumvent this effect by scavenging unreac[ted] diethyl pyrocarbonate by adding excess 2-mercaptoethanol [] s after the start of homogenization were unsuccessful. De[]creasing the initial concentration of diethyl pyrocarbonate on[ly] resulted in partially degraded RNA. These deleterious effe[cts]

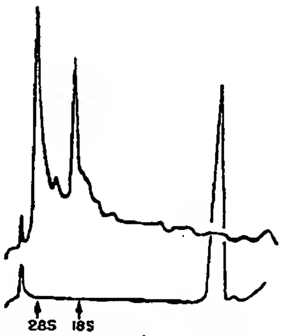

FIGURE 1: Scan at 260 nm of electrophoresis of RNA on 4% acrylamide gel in 98% formamide (Pinder et al., 1974). Direction of migration was from left to right. Upper trace: RNA prepared with guanidinium thiocyanate plus 2-mercaptoethanol by ethanol precipitation. Lower trace: RNA prepared by dropping a pancreas into a blender running at full speed at 4 °C containing 0.1 M sodium acetate, pH 5, 5 mM iodoacetate, 2 mg/mL heparin sulfate, and 0.5% sodium dodecyl sulfate plus an equal volume of buffer-saturated phenol.

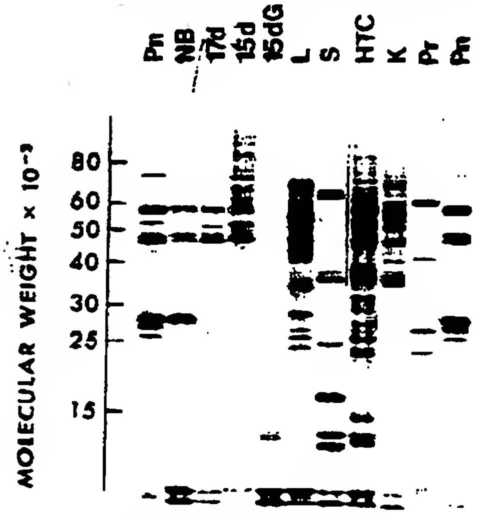

FIGURE 2: Autoradiogram of [^{35}S]methionine-labeled peptides synthesized from purified RNAs in a rabbit reticulocyte lysate (Pelham & Jackson, 1976). Analysis was as described by MacDonald et al. (1977). Total RNA samples (all from rat) were the following: Pn, adult pancreas (24 μg); NB, newborn pancreas (23 μg); 17d, 17-day embryonic pancreas (21 μg); 15d, 15-day embryonic pancreas (21 μg); 15dG, 15-day embryonic gut (22 μg); L, adult liver (40 μg); S, adult submaxillary gland (41 μg); HTC, hepatoma cell line (37 μg); K, adult kidney (27 μg); Pr, adult parotid gland (21 μg).

of diethyl pyrocarbonate led us to develop the improved guanidinium thiocyanate method described here.

The recovery of RNA from adult rat pancreas by the guanidinium thiocyanate procedure (see Experimental Procedures) was ~20 mg/g of wet tissue, varying by 10%. RNA preparations had a A_{260nm}/A_{280nm} ratio of 2.1 or better, indicating low protein contamination. The DNA content was less than 0.5% by the diphenylamine assay (Burton, 1956). Total RNA prepared by this procedure was analyzed by electrophoresis under denaturing conditions in 4% acrylamide gels in 98% formamide (Pinder et al., 1974). Figure 1 shows the absorbance profile of such a gel at 260 nm, demonstrating the presence of intact 28S and 18S ribosomal RNAs in the sample prepared with guanidinium thiocyanate. The lower trace in Figure 1 demonstrates the degradation of these species when isolated by conventional phenol extraction in the presence of a panoply of ribonuclease inhibitors: heparin, iodoacetate, and sodium dodecyl sulfate. Electrophoresis in 3% agarose gels in 6 M urea, pH 3.5 (Woo et al., 1975), gave results (not shown) very similar to those found with formamide gels. Between 1.0 and 1.5% of the total RNA was contained in the polyadenylated fraction after two passages over oligo(dT)–cellulose (Aviv & Leder, 1972).

The products of translation in vitro of RNAs isolated from a variety of rat tissues with guanidinium thiocyanate are displayed in Figure 2. The discrete, tissue-specific products seen for embryonic and adult rat pancreas, liver, kidney, submaxillary, parotid, and HTC cell (a rat hepatoma cell line) RNAs indicate that the guanidinium thiocyanate procedure yields RNA suitable for protein synthesis.

The relatively simple set of proteins synthesized in vitro in Figure 2, lane Pn, suggested that polyadenylated pancreatic RNA should contain a limited number of discrete messages. This was borne out by the profile of oligo(dT)–cellulose-bound RNA on a denaturing agarose–urea gel (Figure 3). When the indicated RNA peaks were eluted from the gel, they were found to be enriched in their template activities for specific polypeptide bands (Figure 4). The major protein band in lane

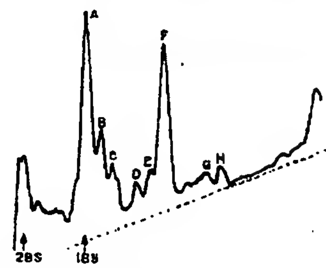

FIGURE 3: Scan at 260 nm of a 3% agarose–6 M urea gel, pH 3.5 (Woo et al., 1975). A 40-μg sample of polyadenylated rat pancreas RNA was run on a 6 × 98 mm gel, which was sliced as indicated.

FIGURE 4: Translational specificity of pancreatic mRNAs resolved by agarose gel electrophoresis. Autoradiogram was prepared as in Figure 2. The in vitro translations were performed on the entire extracted aliquots of RNA from the slices indicated in Figure 3, except for bands A and F of which only half of the aliquot was translated. Lane T contains the translation products of unfractionated pancreatic polyadenylated RNA.

A has been demonstrated to be a precursor (58 000 molecular weight) of α-amylase by specific immunoprecipitation (MacDonald et al., 1977; Przybyla et al., 1979). The RNA coding for amylase (Figure 3, peak A) comigrated with 18S rRNA. On the basis of the in vitro translation data and cDNA–mRNA hybridization complexity (Harding & Rutter, 1978), the isolated amylase mRNA was judged to be greater than 80% pure.

Since it is obviously not possible in this system to compare the quality of the RNAs prepared by the guanidinium thiocyanate and conventional phenol methods, we have performed comparative experiments using dog pancreas, which lacks detectable levels of RNase A (Zendzian & Barnard, 1967). Total polysomal RNA was prepared by conventional phenol–chloroform extraction by MacDonald et al. (1977). The data obtained for dog pancreas were very similar to those shown in Figures 1–3 for rat pancreas. In both species, α-amylase was the dominant tissue-specific protein and its RNA comigrated with 18S rRNA. The patterns of the translation products and of the polyadenylated RNAs were similar. When copied with reverse transcriptase, rat pancreas RNA gave the highest incorporation of nucleotides per gram when the RNA had been prepared with guanidinium thiocyanate (0.13 nmol of [^{3}H]dCMP per μg of polyadenylated RNA) and the lowest when prepared with guanidine hydrochloride plus diethyl pyrocarbonate (0.06 nmol of [^{3}H]dCMP per μg). In comparison, phenol-prepared dog RNA gave an incorporation of 0.11 nmol of [^{3}H]dCMP per μg. All pancreatic cDNAs displayed the same size distribution on alkaline sucrose gradients.

When phenol-extracted dog pancreas RNA was hybridized in excess to cDNAs made from RNA templates prepared either with phenol or guanidine hydrochloride plus 1% diethyl pyrocarbonate, indistinguishable results were obtained. The curves were very similar to those seen for rat pancreas RNA–cDNA hybridizations (Harding et al., 1977).

Discussion

Because of the high concentrations of RNase and RNA in the rat pancreas, polyanionic competitive inhibitors of RNase such as heparin, polyvinyl sulfate, and macaloid (Parish, 1972) cannot be brought practically to high enough concentrations to be useful. Similar limits to attainable concentration preclude the use of antibodies against or protein inhibitors of RNase (Brown et al., 1959; Gribnau et al., 1969). The well characterized covalent inactivators of bovine pancreatic RNase A such as 3-bromopyruvate and iodoacetate react much too slowly to be of use (Heinrikson et al., 1965). Diethyl pyrocarbonate is an effective active-site histidine reagent against pancreatic RNase, but unfortunately this reagent also modifies nucleic acids (Ehrenberg et al., 1974). Such modification may account for the loss of amylase mRNA translation activity and template activity for RNA-directed DNA polymerase described above. Diethyl pyrocarbonate has been reported to destroy ovalbumin message activity (Palmiter, 1974). As demonstrated by Figure 1, phenol plus sodium dodecyl sulfate does not denature RNase sufficiently rapidly to prevent massive degradation of pancreatic RNA.

Although ribonuclease is the bane of molecular biologists, it has been a boon to physical biochemists. It is a thoroughly investigated model of protein denaturation. The transition state for denaturation of pancreatic RNase A is close to the denatured state, so that reagents of increasing effectiveness for equilibrium denaturation will denature with increasing rapidity (Tanford, 1968). Thus, the half-life of RNase is 3 min in 8 M urea (Barnard, 1964) and 10 s in 4 M guanidine hydrochloride (Miller & Bolen, 1978). Both Von Hippel & Wang (1964) and Castellino & Barker (1968) found guanidinium thiocyanate was about 2.5-fold more effec[tive on] a molar basis than guanidine hydrochloride as an equili[brium] denaturant. In the former salt both cation and anion are [a] chaotropes, while in the latter only the guanidinium cat[ion is] chaotropic and hence active in denaturation (Jencks, 19[69]). Thus, it was expected on the basis of the rate dependency [on] denaturant strength that guanidinium thiocyanate would [be] a much more rapid denaturant of RNase than guanidine [hy]drochloride, thus permitting the isolation of intact rat pa[n]creatic RNA without recourse to diethyl pyrocarbonate.

RNA prepared from pancreas with guanidinium thiocyanat[e] can be translated in vitro to give products (Figure 2) very similar to those seen for phenol-isolated dog pancreas RNA (MacDonald et al., 1977) and very similar to the contents of pancreatic secretory granules (Przybyla et al., 1979). Since α-amylase is the major secretory product of the pancreas, its message should be an abundant polyadenylated species. Figures 3 and 4 suggest that this is the case. The 18S polyadenylated RNA (Figure 3, peak A) is the predominant component resolved, although its abundance may be exaggerated by contaminating rRNA. Hybridization between this purified amylase mRNA and the cDNA made from it indicates that the message is more than 80% composed of a sequence of 1500-nucleotide complexity, just large enough to code for the pre-amylase polypeptide (Harding & Rutter, 1978).

In addition to hybridization experiments with purified m[RNA,] pancreatic amylase mRNA, total rat pancreas RNA from adults and developing embryos has been analyzed by cDNA hybridization kinetics (Harding et al., 1978). In no case is there any indication that isolation with guanidinium thiocyanate plus 2-mercaptoethanol introduces any artifactual modifications into RNA or causes the selective loss of any species other than 4S RNA. Recovery of RNA from the organ was quantitative within the experimental uncertainty of such determinations (Schneider, 1946).

RNA prepared with guanidinium thiocyanate from rat islet[s] of Langerhans has been used to synthesize double-stranded cDNA. When this DNA was inserted into a bacterial plasmid, cloned, and analyzed, it was found to contain nucleotide se[quences] correctly coding for the complete amino acid sequen[ce] of rat proinsulin I (Ullrich et al., 1977), thereby confirmin[g] that the information content of the starting RNA was retain[ed] during the procedure.

The predicted utility of guanidinium thiocyanate was full[y] confirmed by the results described above: RNA isolated wi[th] the reagent was physically intact and fully active in translatio[n,] specific message purification, hybridization, and recombina[nt] DNA experiments. The variety of tissues from which activ[e] RNA has been obtained with this method (Figure 2) sugges[ts] that the guanidinium thiocyanate procedure offers a usef[ul] alternative to phenol-based methods, particularly for [ribonu]clease-containing cells.

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Measurement of the Transcription of Nuclear Single-Copy Deoxyribonucleic Acid during Chloroplast Development in *Euglena gracilis*†

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ABSTRACT: The fraction of nuclear single-copy deoxyribonucleic acid (DNA) transcribed at different stages of chloroplast development in *Euglena gracilis* (Z strain) was measured by RNA–DNA hybridization. *Euglena* cells were grown in a heterotrophic medium in the dark to stationary phase and transferred to the light. Total cell RNA was isolated at various stages of chloroplast development and hybridized in a vast excess to ^{125}I-labeled single-copy DNA. The fraction of ^{125}I-labeled single-copy DNA in the form of a duplex was measured by using S1 nuclease. The amount of RNA–DNA hybrid in the duplex mixture was determined by correcting for the contribution of DNA–DNA renaturation. The fraction of single-copy DNA transcribed was calculated by multiplying by 2 the amount of DNA in the form of an RNA–DNA hybrid and correcting for the reactivity of the single-copy DNA probe with total DNA. In dark-grown cells (i.e., prior to the initiation of chloroplast development), the complexity of total cell RNA derived from single-copy DNA was 8.0×10^7 nucleotides. After initiation of chloroplast development, the complexity of the total cell RNA derived from single-copy DNA first increased slightly to 8.9×10^7 nucleotides and then progressively decreased to 7.4×10^7 and 6.4×10^7 nucleotides after 12, 48, and 72 h of exposure to light, respectively. Total cell RNA isolated from cells which had never been cultured in the dark had a complexity of 6.5×10^7 nucleotides.

Chloroplasts are complex organelles which require a multitude of membrane structures, enzymes, and electron-transport constituents to carry out photosynthesis. The development of a functional chloroplast from a proplastid, the progenitor of chloroplasts, presents an interesting example of the need for the coordinate expression and interaction of two distinct genomes within the plant cell. Both the chloroplast and the nuclear DNAs contribute genetic information required for the production of a photosynthetically competent organelle (Schiff,

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